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BRCA2 is a tumor suppressor gene which plays an essential role in cellular proliferation and development. Germline mutations in BRCA2 cause familial early onset breast cancer. BRCA2 gene encodes a protein of 3418 amino acids whose function remains largely unclear. The objective of this study is to analyze the tumor suppressor function of Brca2 gene by manipulating its expression in normal breast cells and breast cancer cells.

Towards this goal, cDNAs corresponding to BRCA2 gene were isolated by screening a Jurkat cDNA library. Northern hybridization analysis of total RNA from normal and cancer cell lines indicated an alteration of transcript size in MCF7 and DU145 cells. Mutational analysis of BRCA2 transcripts from these cell lines is in progress. Full length BRCA2 cDNA was synthesized from a normal breast epithelial cell line, MCF10A using a combination of RT-PCR and cloning strategies. The entire coding sequence was introduced into two mammalian expression vectors (pBKCMV and ptTAS) in both sense and antisense orientation. A second antisense construct encompassing the 5' untranslated sequences and exons 1-7 was designed and cloned in a tetracycline inducible expression vector, ptTAS. These constructs were characterized by restriction fragment analysis and PCR . Expression studies of the corresponding RNAs in transfected normal mammary epithelial cells and breast cancer cell lines is in progress.

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INTRODUCTION

Breast cancer is a major health problem affecting American women, accounting for 181200 new cases diagnosed in 1997 and 17% of all cancer deaths (Parker et al, 1997). Among the various epidemiological factors that contribute to the development of breast cancer, a positive family history of breast cancer in a first degree relative is associated with a doubling of risk (Claus et al, 1990). It has been estimated that genetically inherited forms of breast cancer account for approximately 5%-10% of all breast cancer cases (Weber and Garber, 1997). Genetic linkage analysis in large kindred's with several affected individuals, has localized two breast cancer susceptibility genes, Brca1 (Hall et al, 1990) and Brca2 (Wooster et al, 1994) to the long arm of chromosomes 17q21 and 13q 12 respectively. These two genes have recently been isolated by positional cloning strategies (Miki et al, 1994; Wooster et al, 1995). Germline mutations in Brca1 predispose the carrier females to early onset breast and ovarian cancer, while Brca2 mutations increase the susceptibility to breast and pancreatic cancer. Unlike Brca1, mutations in Brca2 also increase the risk of male breast cancer (Stratton et al, 1994). Identification of these 2 breast cancer predisposing genes has enabled the development of diagnostic tools for carrier detection and therapeutic intervention in familial breast cancer.

Loss of heterozygosity (LOH) studies of polymorphic markers linked to Brca1 and Brca2 loci in breast tumor tissue from affected individuals indicate the loss of the wild type allele, pointing to the function of these 2 genes as tumor suppressors. However, since protein products encoded by either of these genes shows no known sequence similarity to any other protein in the database, deciphering the biological functions of both Brca1 and Brca2 has been rather difficult. The Brca1 gene encodes a protein of 1863 amino acids, containing a RING domain in its N-terminal sequence and a negatively charged region in its C-terminus. There is some evidence that this negatively charged domain may contribute to transcriptional activation of GAL4-Brca2 fusion protein seen in vitro (Monteiro et al, 1996; Chapman et al, 1996). Using immunoprecipitation and cytochemical staining techniques, Brca1 protein was found to co-localize and interact with Rad51 in mitotic and meiotic cells, suggesting a role for this protein in DNA repair (Scully et al, 1997). Loss of Brca1 function by targeted disruption in embryonic cells to generate knockout mice revealed a lethal phenotype resulting from the arrest of cellular proliferation and differentiation (Gowen et al, 1996; Hakem R et al, 1996; Liu CY et al, 1996). These experiments have suggested a developmental role for Brca1 which contrasts with its role as a negative regulator of cell proliferation in tumor suppression.

Brca2 encodes a protein of 3418 amino acids with no significant sequence similarity to Brca1or any other known protein. A portion of BRCA2 protein near the N-terminus encoded by exon 3 has sequence similarity to the transcription activation domain of c-jun. Gene fusion experiments including exon 3 of BRCA2 have revealed stimulation of transcription in yeast and mammalian cells (Milner and Ponder, 1997). BRCA2 knockout mice, likewise show embryonic lethality and developmental arrest due to a defect in cell proliferation (Sharan et al, 1997). Using a yeast two hybrid screen, these authors also demonstrated an association of Brca2 with the DNA repair protein, Rad51. The involvement of Brca2 in the Rad51- dependent double strand break repair pathway correlates well with the observation of increased radiation sensitivity of Brca2-/- embryos in vivo and with hypersensitivity to genotoxic agents in vitro of cultured cells derived from truncated Brca2 mutant embryos (Patel et al, 1998). The precise timing of cell cycle arrest in G1 and G1/M phases with a concomitant increase in the expression of p53 and p21 which signaled DNA damage were postulated to define a role for Brca2 in DNA repair (Patel et al, 1998).

Even though, the above data based on knockout mouse models (Ludwig et al, 1997; Suzuki et al, 1997) explain a plausible function of Brca2 in development, it does not fully address the tumor suppressor role for this gene, the loss of function of which in tumor cells ultimately leads to

increased cellular proliferation. Blocking the expression of Brca2 in differentiated mammary epithelial cells and in breast cancer cell lines using antisense technology to study the resulting phenotype in terms of any alteration in the levels of cell cycle control proteins will be crucial to an understanding of this mammary carcinogenesis pathway and in identifying the key mediators of Brca2 function. Complementary experiments aimed at reversing the malignant phenotype of breast cancer cell lines by introduction of the wild type gene and assaying for its potential to cause tumors in vivo would, besides attesting the role of Brca2 as tumor suppressor, provide a rationale for the development of corrective therapies for specific types of breast cancer. This is particularly important since both radiation and many cytotoxic drugs used currently in the treatment of cancer cause DNA damage and subsequent arrest of cell division, accounting in part for their efficacy. Hence an understanding of Brca2 mechanism could play a decisive role in breast cancer treatment.

EXPERIMENTS AND RESULTS

This project is aimed at addressing the role of Brca2 gene as a tumor suppressor in *in vitro* and *in vivo* assays. As a first step in realizing the aims of this project, I have isolated and cloned copies of the full length Brca2 cDNA and obtained preliminary data on its expression pattern in normal and breast cancer cell lines (Fig. 1). Screening cDNA libraries with Brca2 probes yielded clones corresponding to exon 10, parts of exon 10-11 and 3' untranslated regions. However, the cDNA fragments isolated by screening procedures were not contiguous and hence the complete sequence of Brca2 could not be assembled from these partial cDNAs. I have successfully used RT-PCR to synthesize full length Brca2 cDNAs from a normal breast epithelial cell line, MCF10A. This cDNA has been cloned into a mammalian expression vector, pBKCMV in both the sense and antisense orientations.

An alternate antisense construct encompassing the 5' untranslated region (UTR) plus exons 1-7 of Brca2 gene was designed and cloned in ptTAS, a tetracycline inducible mammalian expression vector. This construct besides providing a dose dependent expression of antisense RNA can also be used in rescue experiments in which restoration of Brca2 function will be tested by the introduction of plasmid containing only the coding sequence. Thus, I have designed and constructed all the necessary reagents to test the aims of this project. The plasmid DNAs have already been transfected into normal breast epithelial and breast cancer cell lines and work is in progress to obtain stable transformant clones.

1.Construction of full length Brca2 cDNA:

Two different strategies were employed to generate full length Brca2 cDNAs. a) Screening a cDNA library and b) Long-Accurate (LA) PCR.

A. Jurkat cDNA library screening

First, a Jurkat cell cDNA library in lambda phage was screened using a 32 P-labeled Brca2 clone obtained previously in our laboratory in the course of isolating transcribed sequences from the chromosomal region 13q12 which harbors the Brca2 gene (Jacob et al, 1996). Nearly 1 million plaques were screened and 7 positive clones were obtained. The sizes of insert DNAs in these clones were approximately 1.2 –2.0 kb and restriction digestion analysis indicated they all represented the same region on the 3' end of the mRNA. Dye deoxy sequencing was performed on one of the clones using an automated ABI sequencer and the results indicated an authentic Brca2 sequence.

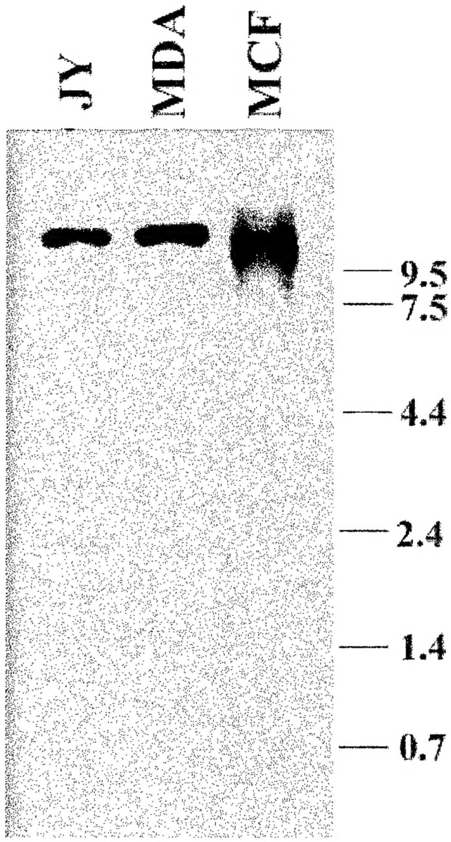


Fig. 1 Northern Blot analysis of total RNA isolated from a normal lymphoblastoid cell line (JY), and two breast cancer cell lines (MDA-MB468 and MCF7) using radio-labeled Brca2 cDNA probe. The numbers on the right denote sizes (in kb) of RNA markers.

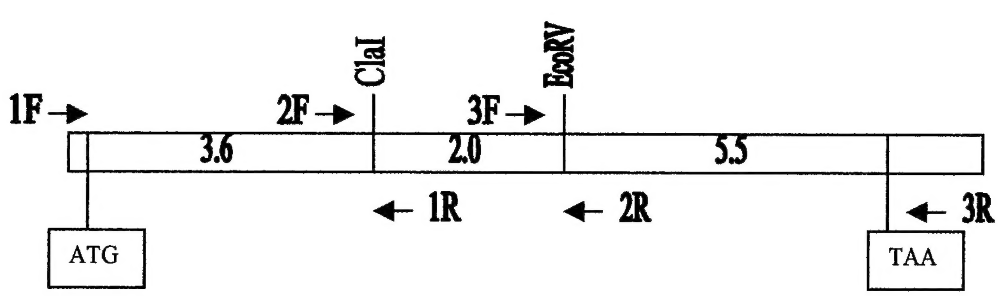
Since the 3' probe did not detect additional clones corresponding to upstream regions of the mRNA from this library, I synthesized a 5' probe using PCR primers flanking exon 10 of BRCA2 gene. The PCR product was sequenced to confirm authenticity and used as a probe to fish out additional clones for the 5' region of Brca2 from Jurkat cDNA library. Analysis of 15 positive clones obtained from this screening gave insert DNAs ranging in size from 1.5-2.0kb. Further restriction analysis revealed that they represented the same region of the mRNA, corresponding to exon 10 of Brca2 gene. This high redundancy and lack of contiguous cDNA fragments could be attributed to the higher size (11Kb) of Brca2 transcript, reducing the probability of obtaining a full length clone from this unfractionated cDNA library. Therefore it was not possible to assemble the complete cDNA by this approach.

B. RT-PCR amplification of full length Brca2 cDNA:

Northern blot analysis of total RNA from normal breast epithelial cells and breast cancer cell lines have shown fairly high abundance of Brca2 mRNA in the desired size range (11-12kb) to obtain full length cDNA. Total RNA was isolated from MCF10A cells grown to 70% confluency, using RNAzol method. Poly A(+) RNA was purified using oligotex beads (Qiagen). Total RNA (10μg) or 500ng of polyA(+) RNA was used as template for first strand cDNA synthesis with oligo dT primer and Superscript (Gibco-BRL) reverse transcriptase. The reaction was performed at 48°C to prevent the formation of secondary structure and to facilitate synthesis of longer full length cDNA. An aliquot (5μl) of this cDNA was used in LA-PCR using Brca2 gene-specific primers and Expand High Fidelity Taq polymerase mixture (Boehringer Mannheim). Two strategies were employed for PCR amplification to generate a complete cDNA.

I. Amplification of three overlapping fragments followed by ligation at unique restriction enzyme sites:

The published sequence of Brca2 gene was analyzed for the presence of unique restriction enzyme sites using mapplot program in GCG package. Brca2 mRNA is 11,319 nt long containing an open reading frame (229 – 10482) of 10254 nt. A unique ClaI site at nt 3742 and a unique EcoRV site at nt 5512 were identified as suitable points for designing flanking oligonucleotide primers. Three sets of primers were designed such that PCR amplification would produce fragments of sizes 3.6, 2.0 and 5.5kb corresponding to the 5', middle and 3' portions of the mRNA. These fragments would then be sequentially introduced into the multiple cloning site of the expression vector pBKCMV. A schematic representation of this strategy is shown below.



The following oligonucleotide primers were synthesized for this purpose:

1F: 5' CTCTGGAGCGGACTTATTTACC (178-200 nt)
1R: 5' GCTTGCTGCTGTCTACCTGACC (3769-3748 nt)
2F: 5' CCAAGCTACATATTGCAGAAGAG (3625-3647 nt)
2R: 5' CAACGCAAATATCTTCATTTACAG (5659-5636 nt)
3F: 5' CCTTGAATTAGCATGTGAGACCAT (4953-4976 nt)
3R: 5' TCGCCTTTGCAAATGCTTAG (10501-10482 nt)

PCR was performed with pairs of these primers and 5μl each of first strand cDNA using the Expand High Fidelity PCR System (Boehringer Mannheim). The reaction mixture contained 1X buffer, 1.5mM $MgCl_2$, 200μM each of dATP, dCTP, dGTP, dTTP, 300nM each of the forward and reverse primers, 2.6 U of enzyme mix and the template DNA in a total volume of 50μl. The enzyme and dNTPs together were added after the reaction mixture reached 80°C to initiate a "hot start" reaction. The thermal cycling was done in a Perkin –Elmer 9600 PCR machine using the following conditions.

Initial denaturation at 94°C, 2min followed by 10cycles of
Denaturation at 94°C, 15s
Annealing at 62°C, 30s
Elongation at 68°C, 4min followed by 20 cycles of
Denaturation at 94°C, 15s
Annealing at 62°C, 30s
Elongation at 68°C, 4min + an autoextension of 5s for each cycle

and a final elongation cycle at 72°C for 7 min. An aliquot (7μl) of the reaction product was analyzed by 0.8% agarose gel electrophoresis and the DNA bands were visualized by ethidium bromide staining (Fig. 2). Amplified DNA fragments of the expected size were obtained. Since the template cDNA was oligo

dT primed, this result indicated to the possibility of obtaining the full length molecule by PCR. In parallel with these experiments, LA-PCR was also performed using an aliquot of the same first strand cDNA.

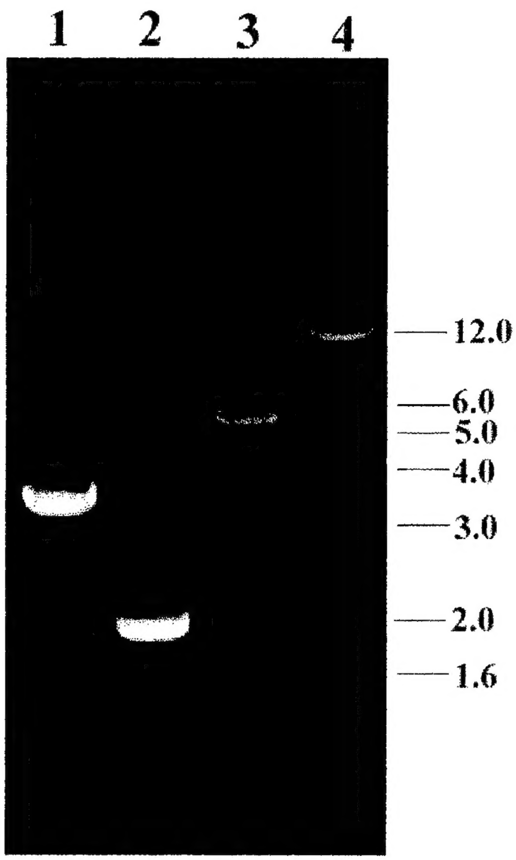


Fig. 2. High Fidelity RT-PCR amplification of Brca2 cDNA fragments using oligonucleotide primer set 1F/1R (lane 1), 2F/2R (lane 2) and 3F/3R (lane 3).

II. Long-Accurate PCR (LA-PCR) amplification of full length Brca2 cDNA:

LA-PCR was performed on 5μl of the first strand cDNA sample with primers 1F and 3R using Expand High Fidelity PCR system (Boehringer Mannheim). An alternate forward primer, brex1F (5'AGATCTGTGGCGCGAGCTTCTGAAACTAG), designed to include 5' untranslated region of the Brca2 mRNA was also used along with the reverse primer (3R) in a separate PCR reaction. The reaction mixture was essentially identical to the one described above. The amplification conditions, however, were chosen to facilitate longer extension times as follows:

Initial denaturation at 94°C, 2min followed by 10cycles of
Denaturation at 94°C, 15s
Annealing at 62°C, 30s
Elongation at 68°C, 8min followed by 20 cycles of
Denaturation at 94°C, 15s
Annealing at 62°C, 30s
Elongation at 68°C, 8min + an autoextension of 10s for each cycle

and a final elongation cycle at 72°C for 7 min. An aliquot (7μl) of the reaction product was analyzed by 0.8% agarose gel electrophoresis and the DNA bands were visualized by ethidium bromide staining (Fig. 3).

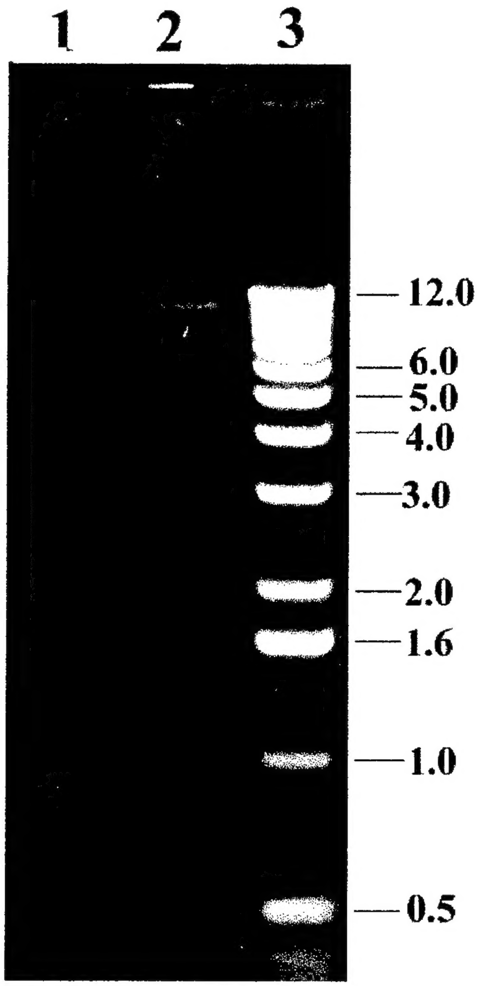


Fig. 3. RT-PCR amplification of full length Brca2 cDNA using mRNA from MCF10A cells and Expand Hi Fi System. Lane1: brex1F/3R primer pair. Lane2: 1F/3R primer pair.

Both pairs of primers gave an amplified product of the expected size, 10.5kb (Fig. 4 Lanes 1 and 2). The remainder of the reaction product was extracted with phenol, phenol:chloroform, and the DNA was precipitated with sodium acetate and ethanol. This purified DNA was blunt ended and cloned first into pBluescript and subsequently into the mammalian expression vector, pBKCMV. Cloning in the EcoRV site of pBluescript facilitated a wider choice of restriction sites on either side for subsequent directional cloning into the expression vectors. Blunt end ligations were performed with digested vector after polishing the ends of the PCR product with Klenow polymerase. The ligated plasmids were transformed into E.coli, DH5α and plated on selective media. Twenty white colonies were picked at random, resuspended each in 50μl of LB and 1μl of the suspension was used as template for colony PCR using gene specific internal primers (1F and brex7R). Analysis of the PCR product by 1% agarose gel electrophoresis showed the presence of Brca2 inserts in 3 independent clones (Fig. 4). These positive clones were grown in LB medium and plasmid DNA was isolated using Qiagen miniprep kit. Restriction digestion analysis of the plasmid DNAs with NotI and SalI released the insert of the expected size

(10.5kb) from one of the PCR-positive clones. The orientation of the insert DNA within the vector sequences was inferred from experiments using the flanking vector primers (T3 or T7) in conjunction with a gene specific internal primer (brex7R) to amplify the desired product.

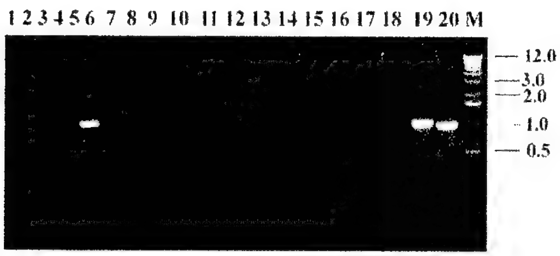


Fig. 4. PCR screening of recombinant colonies using gene specific primers.

The authenticity of this clone (pBrcfl-6) was verified by sequencing the ends of the insert DNA using T3 and T7 primers and by restriction digestion analysis. A representative digestion with BamHI and NotI/SalI double digest is shown in Fig. 5. As expected , BamHI cuts the insert DNA into fragments of sizes 0.5, 2.5 and 7.5kb along with a vector band of 3kb. This full length Brca2 clone is being further characterized with respect to its sequence integrity and parallel experiments are in progress to introduce an expression construct of this clone into breast cancer cell lines MCF7 and MDA-MB468 in order to obtain stable constitutive expression of Brca2 protein.

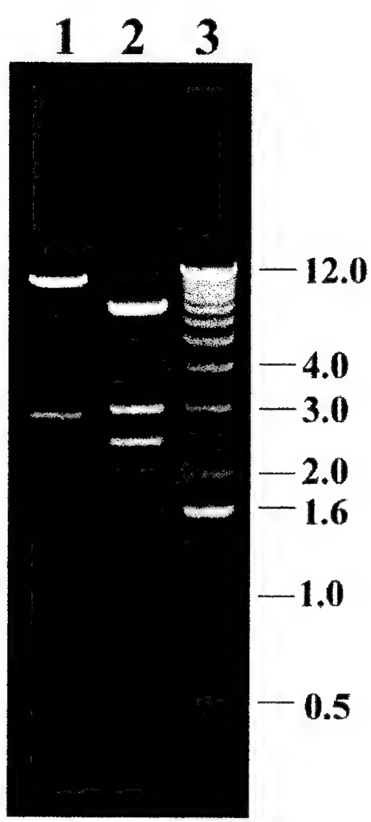
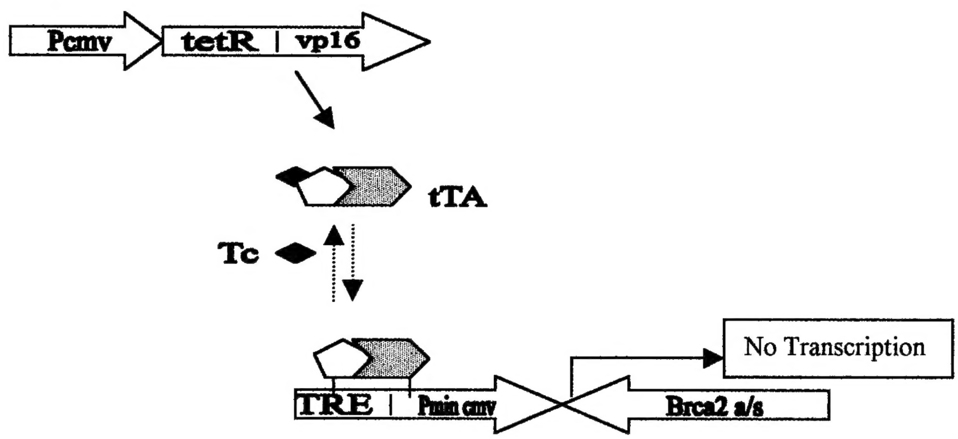


Fig. 5. Restriction digestion analysis of clone pBrcfl-6 with BamHI (Lane 1) and NotI/SalI (Lane 2). Lane 3 represents 1kb ladder.

2. Construction of 5' antisense Brca2 expression plasmid in a Tet-inducible vector, ptTAS:

The recent development of tetracycline-regulated, inducible expression systems have enabled precise and reversible control of exogenous gene regulation in mammalian cells (Shockett and Schatz, 1996). Tet-On and Tet-Off systems offer the advantage of precise temporal and quantitative control of gene expression by simply varying the concentration tetracycline or its derivative doxacycline in the medium, in a dose-dependent manner. These systems are based on the mechanism of the tet-operon of E.coli and basically contain two components (Gossen and Bujard, 1992): 1. The regulator plasmid (pTet-On / pTet-Off) expressing a hybrid protein of theTet repressor fused with the VP16 activation domain of herpes simplex virus. This hybrid protein (tTA) when bound to the tet-operator sequence stimulates transcription from a downstream promoter. 2. A response plasmid (pTRE) which contains a tet-responsive-element placed upstream of a minimal promoter. The gene to be expressed is placed downstream of this promoter. The two plasmids are then stably integrated into the host genome to generate a "double-stable" cell line. In the Tet-Off system the constitutively expressed tTA binds to the TRE and turns on transcription of the exogenous gene. When tetracycline is added to the medium it binds the transactivator, thereby preventing its interaction with the TRE and switching off transcription. Decreasing the concentration of tetracycline increases the expression of the transfected gene in dose dependent manner. A schematic diagram of the Tet-Off system is illustrated below.



A modified Tet-Off vector incorporating tTA and TRE in the same plasmid, ptTAS (gift from Dr. Clement Lee) was used to construct a 5' Brca2 antisense expression vector. This vector was chosen since it required only one round of transfection to yield a stable cell line. The cDNA corresponding to exons 1-7 of Brca2 gene was amplified by RT-PCR using a nested set of reverse (brex10R and brex7R) primers and brex1F as the forward primer. The oligonucleotide sequences are :

Brex10R: 5' GGCATTGACTTTCCAATGTGGTC
Brex7R: 5' ACTGAATTCCAGGATCCACCTCAGCTCCTAG

Total RNA (10μg) was reverse transcribed using brex10R as the gene specific primer and Superscript enzyme (Gibco-BRL) as described previously. An aliquot (5μl) of this reaction product was then amplified using the internal brex7R and brex1F as primers. A nested primer set was used for cDNA synthesis and subsequent PCR in order to increase the specificity of amplification. A specific PCR product of the expected size (800bp) was obtained (Fig. 6).

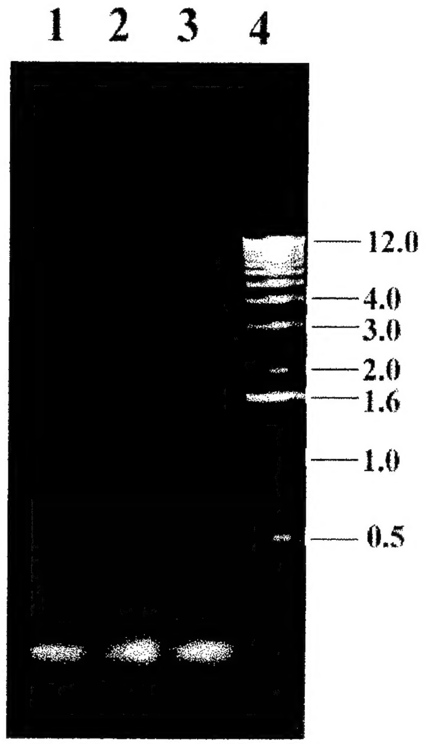


Fig. 6. RT-PCR amplification of Brca2 exons 1-7 using brex1F and a nested set of reverse primers. Lane 1: total RNA primed with oligo dT, Lane 2: total RNA primed with brex10R, Lane 3: poly A RNA primed with brex10R.

The product was gel purified and digested with EcoRI to generate one cohesive end and ligated to EcoRI/BamHI double digested ptTAS plasmid DNA. After ligation, the termini were blunt ended using Klenow polymerase and then recircularized. Ligated DNA was transformed into DH5α competent cells and plated on ampicillin LB-agar plates. Twenty colonies were picked at random and checked for the desired insert by colony PCR as described previously. Three positive clones were obtained. Plasmid DNAs were isolated from these clones and characterized by restriction analysis for authenticity of the clones. The antisense orientation of the insert DNA was confirmed by PCR using CMV promoter primer and Brca2 specific primers (Fig. 7). One of the positive clones, pTBrcut-1 was sequenced for additional confirmation. This DNA along with a another cotransfection plasmid containing a selectable marker will be electroporated into MCF10A cells to obtain a stable cell line expressing Brca2 antisense transcript under tetracycline control.

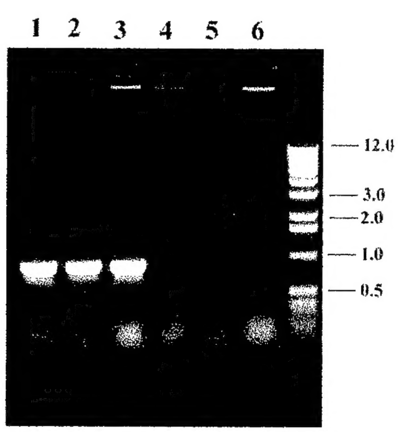


Fig. 7. PCR analysis of pTBrcut clones with CMV promoter primer and brex1F (lanes 1-3) and brex7R (lanes 4 -6).

Summary and Conclusions:

The proposed aims of this project were : 1. to obtain a full length Brca2 cDNA through a variety of strategies and clone it in a mammalian expression vector. 2. To introduce Brca2 expression plasmids into normal and breast cancer cell lines and obtain stable clones. 3. To study the phenotype of cells expressing the sense and antisense RNA by in vitro assays and 4. To test the tumorigenic potential of the transfected cells in vivo using the nude mice model.

This report details the accomplishment of tasks 1-3 of the first technical objective, namely , constructing a full length cDNA for Brca2 gene and cloning it in an expression vector. The extremely high size of Brca2 mRNA posed some initial problems in isolating a full length cDNA clone by library screening. Smaller clones corresponding to portions of the Brca2 gene were isolated from a jurkat cDNA library. However these short fragments were incontiguous and hence could not be assembled to generate the entire Brca2 cDNA. Construction of two full length clones (differing in the extent of 5' untranslated sequences) was accomplished by RT- PCR using sets of Brca2 specific primers and the LA-PCR system. The complete cDNAs were cloned in a mammalian expression vector, pBKCMV and characterized by restriction digestion analysis and partial sequencing. An antisense expression plasmid encompassing Brca2 exons 1-7 was designed and engineered in the tetracycline regulated expression system, ptTAS. Experiments are in progress to obtain normal and breast cancer cell lines stably expressing either the sense or antisense Brca2 RNA. Preliminary data on the expression profile of Brca2 mRNA in normal and breast cancer cell lines have been obtained and screening for different mutant cell lines is in progress.

In conclusion, I have generated full length Brca2 cDNA and engineered it in expression plasmids for introduction into normal and breast cancer cell lines. Thus, all the necessary reagents for addressing the technical objective 2 are ready and work in this regard is already in progress.

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